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## Food-derived opioid peptides inhibit cysteine uptake with redox and epigenetic consequences<sup>☆</sup>

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### Abstract

Dietary interventions like gluten-free and casein-free diets have been reported to improve intestinal, autoimmune and neurological symptoms in patients with a variety of conditions; however, the underlying mechanism of benefit for such diets remains unclear. Epigenetic programming, including CpG methylation and histone modifications, occurring during early postnatal development can influence the risk of disease in later life, and such programming may be modulated by nutritional factors such as milk and wheat, especially during the transition from a solely milk-based diet to one that includes other forms of nutrition. The hydrolytic digestion of casein (a major milk protein) and gliadin (a wheat-derived protein) releases peptides with opioid activity, and in the present study, we demonstrate that these food-derived proline-rich opioid peptides modulate cysteine uptake in cultured human neuronal and gastrointestinal (GI) epithelial cells via activation of opioid receptors. Decreases in cysteine uptake were associated with changes in the intracellular antioxidant glutathione and the methyl donor S-adenosylmethionine. Bovine and human casein-derived opioid peptides increased genome-wide DNA methylation in the transcription start site region with a potency order similar to their inhibition of cysteine uptake. Altered expression of genes involved in redox and methylation homeostasis was also observed. These results illustrate the potential of milk- and wheat-derived peptides to exert antioxidant and epigenetic changes that may be particularly important during the postnatal transition from placental to GI nutrition. Differences between peptides derived from human and bovine milk may contribute to developmental differences between breastfed and formula-fed infants. Restricted antioxidant capacity, caused by wheat- and milk-derived opioid peptides, may predispose susceptible individuals to inflammation and systemic oxidation, partly explaining the benefits of gluten-free or casein-free diets.

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**Keywords:** Glutathione; Casomorphin; Gliadin; Autism spectrum disorder; Schizophrenia; Celiac disease; Gluten-free/casein-free diet

### 1. Introduction

Gluten-free or casein-free diets have been reported to improve intestinal, autoimmune and neurological symptoms in celiac disease [1,2], autism [3,4] and schizophrenia [5,6], but the underlying mechanism of benefit for such diets remains unclear. Emerging evidence from biochemical investigations indicate that neurological disorders like schizophrenia and autism spectrum disorder (ASD) might have underlying defects in prenatal and early postnatal neurodevelopment as a contributing factor to the etiology of these disorders. Especially, systemic oxidative stress has been strongly

reported in schizophrenic [7] and ASD [8] patients, associated with significantly lower levels of the antioxidant glutathione (GSH), whose synthesis is limited by cysteine availability. Consistently, brain GSH levels are also reported to be decreased in autism [9] and schizophrenia [10], strongly supporting the proposal that a deficit in this specific antioxidant, namely GSH, might contribute to neurodevelopmental and neuropsychiatric disorders. Notably, intestinal mucosa and plasma levels of GSH are also reduced in pediatric celiac disease patients [11]. Moreover, in few cases, patients with neurological conditions [12] like autism [13] and schizophrenia [14] also report a co-morbid problem of celiac disease and/or gut inflammation.

Oxidative stress and DNA methylation changes are metabolically coupled via the transsulfuration pathway and one-carbon metabolism. DNA methylation is carried out by a class of enzymes called DNA methyltransferases DNMTs, which depend on the levels of S-adenosylmethionine (SAM) [15], which in turn are dependent on the action of the enzyme methionine synthase (MS) and the redox status of the cell [16]. SAM acts a methyl donor for over 200 methylation reactions and is converted to S-adenosylhomocysteine (SAH), an inhibitor of methylation reactions. Hence, the ratio SAM/SAH is termed as the methylation capacity of the cell. Several studies report decreased

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levels of SAM and methylation capacity in patients with ASD and schizophrenia. DNA methylation is an important methylation reaction, involving addition of a methyl groups at CpG sites on DNA, and it is a major factor for epigenetic-based changes in gene expression. In recent studies, abnormal DNA methylation patterns have been implicated in schizophrenia [17,18] and ASD [19,20], but more prominently in the latter. Rett syndrome, immunodeficiency, centromeric instability, facial anomalies (ICF) syndrome and autosomal dominant cerebellar ataxia are few examples of a heterogeneous group of neurodegenerative and neurodevelopmental disorders caused by changes in proteins that either “read” or “write” DNA methylation, resulting in disordered DNA methylation at numerous single-copy genes as well as in specific sequences of repeat DNA in parts of constitutive heterochromatin [17]. Similarly, deficiency of B vitamins, methionine, folic acid and/or choline can significantly alter DNA methylation by affecting levels of SAM and SAH [21,22]. However, while these studies indicate the interaction between nutrients and the pathways regulating DNA methylation, it is still unclear how a specific gene is targeted by nutrients or alternatively, if these changes are stochastic in nature [21,23].

Epigenetic programming during early postnatal development can influence lifelong disease risk [24,25] and may be modulated by nutritional factors, especially factors affecting DNA methylation [26,27]. Milk (either human or bovine) or milk-derived products may be the only source of nutrition for infants during early postnatal development, while wheat-containing foods are commonly introduced after the first year. Casein is an important milk protein, and similarly gliadin is a wheat gluten-derived protein, and hydrolytic digestion of both releases peptides with opioid activity [28,29]. Enzymatic digestion of human  $\beta$ -casein liberates the heptapeptide h $\beta$ -casomorphin-7 (YPFVEPL), designated hBCM7, while bovine  $\beta$ -casein yields b $\beta$ -casomorphin-7 (YPPGGL; bBCM7), and  $\alpha$ -gliadin yields gliadinomorphin-7 (YPQPQPF; GM7) (Fig. 1a). Divergent amino acids at positions 3–5 result in distinctive conformations, and the presence of 2 or 3 proline residues makes these peptides resistant to further proteolysis [30]. Each of these homologous proline-rich peptides has the capacity to activate opioid receptors [28–31].

Endogenous opioid peptides normally found in the body are called endorphins (e.g.,  $\beta$ -endorphins, enkephalins), whereas exogenous opioid peptides (e.g., morphine, hBCM7, bBCM7 and GM7) are called exorphins. Low energy conformations of the food-derived exorphins bear a structural similarity to morphine (Supplementary Fig. 1), particularly at their N-terminal tyrosine residue, which is also a feature of endorphins and is critical for their affinity and efficacy at opioid receptors [32]. Studies show that morphine-induced activation of  $\mu$ -opioid receptors induces changes in the expression of EAAT3, which provides the primary pathway for cysteine uptake in both mature neurons [33,34] and GI epithelial cells [33,35], particularly in the distal ileum [35]. Morphine can induce oxidative stress and cause a pro-oxidant shift in cell redox status [36–38], which subsequently can cause a decrease in the availability of SAM for more than 200 methylation reactions, including DNA methylation, resulting in subsequent transcriptional changes. Hence, the current study investigated whether morphine and food-derived opioid peptides can induce alterations in cellular redox status, DNA methylation [39] and transcription by altering cysteine uptake in SH-SY5Y cells, providing biochemical evidence that could potentially support the use of nutritional intervention, i.e., a gluten-free/casein-free diet.

## 2. Materials and methods

### 2.1. Materials

Minimum essential medium,  $\alpha$ -modification ( $\alpha$ -MEM), trypsin, Hank's balanced salt solution (HBSS) and penicillin/streptomycin/fungizone antibiotic solution were purchased from Mediatech (Manassas, VA, USA). Fetal bovine serum (FBS) was

obtained from Hyclone (Logan, UT, USA). [ $^{35}$ S]-Cysteine was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Morphine and other drugs were obtained from Sigma Chemicals (St. Louis, MO, USA). Human and bovine forms of BCM-7, as well as gliadin-7 peptide, were custom synthesized by Neopeptide (Cambridge, MA, USA).

### 2.2. Cell culture

Monolayers of cells were grown in 10-cm tissue culture dishes with 10 ml of  $\alpha$ -MEM supplemented with 1% penicillin/streptomycin/fungizone and 10% FBS, in 5% CO<sub>2</sub> at 37°C. For most experiments, cells were plated and incubated for 48 h prior to use.

### 2.3. Cysteine uptake

The cysteine uptake assay was performed according to a method described previously [39]. In brief, SH-SY5Y human neuroblastoma cells plated in six-well plates were pretreated with drugs and incubated for various times prior to measuring uptake. Media was aspirated and cells were washed with 600  $\mu$ l of HBSS at 37°C. Non-radioactive HBSS was aspirated, replaced with 600  $\mu$ l of 37°C HBSS containing [ $^{35}$ S]-cysteine (1  $\mu$ Ci/1 ml), 10  $\mu$ M unlabelled cysteine and 100  $\mu$ M DTT, and cells were incubated for 5 min. The [ $^{35}$ S]-cysteine/HBSS mixture was aspirated and treatment was terminated by two washes with ice-cold HBSS. Cells were then lysed with 600  $\mu$ l of dH<sub>2</sub>O, scraped, collected in 1.5 ml microcentrifuge tubes and sonicated for 10 s. One-hundred microliters of each sample was aliquoted for protein assay. Two-hundred microliters of each sample (in triplicate) was aliquoted into scintillation vials with 4 ml of scintillation fluid, vortexed and counted for radioactivity, normalized against protein content.

### 2.4. Thiol metabolite assay

SH-SY5Y neuroblastoma cells were grown to confluence in  $\alpha$ -MEM. Media was aspirated and the cells were washed twice with 1 ml of ice-cold HBSS. HBSS was aspirated and 0.6 ml ice-cold dH<sub>2</sub>O was added to the cells. Cells were scraped from the flask/dish and suspended in dH<sub>2</sub>O. The cell suspension was sonicated for 15 s on ice and 100  $\mu$ l was used to determine protein content. The remaining lysate was added to a microcentrifuge tube and an equal volume of 0.4 N perchloric acid was added, followed by incubation on ice for 5 min. Samples were centrifuged at 5000g and the supernatant was transferred to new microcentrifuge tubes. One-hundred microliters of sample was added to a conical microautosampler vial and kept at 4°C in the autosampler cooling tray. Ten microliters of this sample was injected into the HPLC system.

The separation of redox and methylation pathway metabolites was accomplished using an Agilent Eclipse XDB-C8 analytical column (3 $\times$ 150 mm; 3.5  $\mu$ m) and an Agilent Eclipse XDB-C8 (4.6 $\times$ 12.5 mm; 5  $\mu$ m) guard column. Two mobile phases were used: Mobile Phase A was 0% acetonitrile, 25 mM sodium phosphate and 1.4 mM 1-octanesulfonic acid, adjusted to pH 2.65 with phosphoric acid. Mobile Phase B was 50% acetonitrile. The flow rate was initially set at 0.6 ml/min and a step gradient was used: 0–9 min, 0% B; 9–19 min, 50% B; 19–30 min, 50% B. The column was then equilibrated with 5% B for 12 min prior to the next run. Temperature was maintained at 27°C. The electrochemical detector was an ESA CoulArray with BDD Analytical Cell Model 5040 and the operating potential was set at 1500 mV. Sample concentrations were determined from the peak areas of metabolites using standard calibration curves and ESA-supplied HPLC software. Sample concentrations were normalized against protein content. In some cases, samples were diluted in mobile phase as needed or up to 50  $\mu$ l of sample was injected to assure that thiol levels were within the range of the standard curve.

### 2.5. DNA methylation analysis

Genomic DNA was extracted from samples with the Easy DNA Kit (Invitrogen K1800-01) using the appropriate protocol for cell lines. For a full overview of the MethylCap-Seq protocol, refer to De Meyer *et al.* [40]. In summary, fragmentation was performed on Covaris S2 with the following settings: duty cycle, 10%; intensity, 5; cycles per burst during 200 s, 200; to obtain fragments with an average length of 200 bp. The power mode is frequency sweeping, temperature is 6–8°C and water level is 12. A maximum of 5  $\mu$ g is loaded in 130  $\mu$ l Tris-EDTA in a microtube with AFA intensifier. For samples with less DNA input (down to 500 ng), we dilute the DNA in 1:5 diluted Tris-EDTA. DNA with an input from 5 to 3  $\mu$ g is analyzed on the Agilent 2100 on a DNA 1000 chip. DNA with an input lower than 3  $\mu$ g is concentrated in a rotary evaporator to 25  $\mu$ l and the fragment distribution is checked on a high-sensitivity DNA chip. Methylated DNA was captured using the MethylCap kit (Diagenode, Belgium), according to the manufacturer's protocol; starting concentration was 200 ng. The yield was typically between 0.5 and 8 ng total captured DNA. Fragments were subsequently sequenced using the Illumina Genome Analyzer II. The concentrations of fragmented and captured DNA were determined on a Fluostar Optima plate reader with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen P7589) at 480/520 nm.

To prepare the DNA library, we used the DNA Sample Prep Master Mix Set 1 (NEB E6040) in combination with the Multiplexing Sample Preparation Oligo Kit (96 samples, Illumina PE-400-1001). We use the entire fragmented DNA and followed the NEB protocols. NEBNext End Repair Module Protocol is to purify with a Qiagquick PCR Purification Kit (Qiagen 28104) and elute in 37  $\mu$ l Elution Buffer (EB). NEBNext dA-tailing Module Protocol is to purify on a minelute PCR Purification Kit (Qiagen 28004) and elute in 25  $\mu$ l EB. NEBNext Quick Ligation Module Protocol is to purify on a minelute

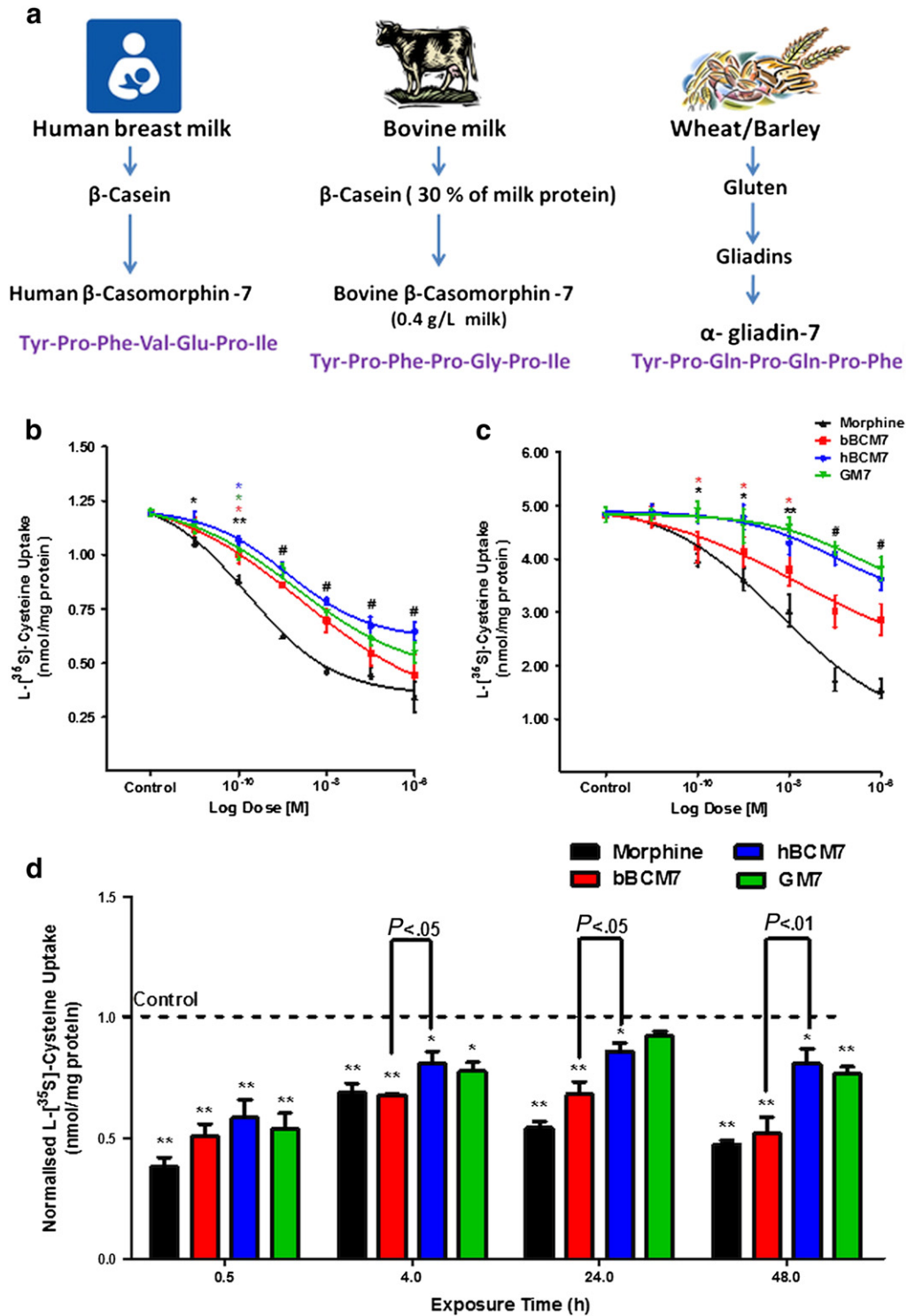


Fig. 1. Food-derived opioid peptides and morphine inhibit cysteine uptake in SH-SY5Y cells. (a)  $\beta$ -Casein from milk and  $\alpha$ -gliadin from wheat, barley and rye are hydrolysed in the intestine by pepsin, leucine aminopeptidase and elastase to release peptides with opioid activity. The 7-aa proline-containing peptides from human and bovine milk, and that from wheat, have homologous sequences, including two or three proline residues. (b) Radiolabeled cysteine uptake in SH-SY5Y human neuroblastoma and (c) Caco2 colon carcinoma cell lines in the presence of increasing concentrations (0.1 nM to 1  $\mu$ M) of morphine or opioid peptides. Cells were treated for 30 min. (d) Time-dependent effects of opioid peptides and morphine on cysteine uptake by SH-SY5Y cells. SH-SY5Y cells were treated with 1  $\mu$ M of morphine or opioid peptides for 0.5, 4, 24 and 48 h (n=6). \* $P$ <.05 and \*\* $P$ <.01 vs. control.

PCR Purification Kit (Qiagen 28004) and elute in 30  $\mu$ l EB, using the multiplexing sequencing adapters provided in the Multiplexing Sample Preparation Oligo Kit. Size selection of the library was done on a 2% agarose gel (Low Range Ultra Agarose Biorad 161-3107). We used a 1-Kb Plus ladder (Invitrogen 10787-018) and ran the gel at 120 V for 2 h. A fragment of 300 $\pm$ 50 bp was excised and eluted on a Qiagen Gel Extraction Kit column (Qiagen 28704) and eluted in 23  $\mu$ l EB.

We followed the Illumina library amplification index protocol with the following alterations: We used 22  $\mu$ l DNA and performed 21 cycles. The sample was purified on a Qiaquick PCR Purification column (Qiagen 28101) and eluted in 50  $\mu$ l EB, 1:5 diluted and concentrated in a rotary evaporator to 10  $\mu$ l, and 1  $\mu$ l was applied to an Agilent 2100 HS DNA chip and the concentration was determined by smear analysis on the Agilent 2100. The samples were diluted to 10 nM. After denaturation with NaOH, we diluted

the samples to 16 pM. The paired-end flow cell was prepared according to the Cluster Station User Guide. Sequencing was performed according to the HiSeq user guide (performing a Multiplexed PE Run), with 2×51 cycles for the paired-end runs.

### 2.6. Statistical methods

Statistical analyses were carried out using GraphPad Prism version 5.01. Student's *t* test for independent means was used to test for significant differences between control and experimental groups. Data were expressed as mean±standard error of the mean. Best-fit values were calculated using nonlinear and linear regression models. Nonlinear regressions used a two-phase exponential decay function. Comparisons between multiple groups of data were conducted using one-way analysis of variance, and Tukey's *post hoc* test was used to determine the differences between individual groups.

### 3. Results

We previously characterized EAAT3 expression in SH-SY5Y human neuroblastoma cells and indicated that it makes a major (>90%) contribution to [<sup>35</sup>S]-cysteine uptake [39]. To investigate whether morphine and opioid peptides could alter EAAT3 activity via  $\mu$ -opioid receptors, we carried out a radiolabeled [<sup>35</sup>S]-cysteine uptake assay in SH-SY5Y cells. Pretreatment of SH-SY5Y human neuroblastoma cells with either morphine or casein/gluten-derived exorphin peptides for 30 min inhibited the uptake of radiolabeled [<sup>35</sup>S]-cysteine in a concentration-dependent manner, with an efficacy order of

morphine>bBCM7>hBCM7>GM7 and IC<sub>50</sub> values of 0.16, 1.31, 4.34 and 1.94 nM, respectively (Fig. 1b). Morphine and exorphin peptides inhibited cysteine uptake with similar efficacy but lower potency in Caco2 cells, a cell line derived from human GI epithelial cells, with IC<sub>50</sub> values of 6.38, 15.95, 90.08 and 301 nM, respectively (Fig. 1c). Inhibition of cysteine uptake was fully developed at 30 min and was sustained through 48 h of morphine or exorphin exposure, although the extent of inhibition was modestly decreased in a time-dependent manner. Inhibition by bBCM7 was greater than that of hBCM7 at 4, 24 and 48 h (Fig. 1d). Cysteine uptake effects of morphine and exorphin peptides were blocked by naltrexone, an antagonist of both  $\mu$ - and  $\delta$ -opioid receptors (Supplementary Fig. 2), as well as by D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr (CTAP), a selective  $\mu$ -antagonist, but not by the  $\delta$ -opioid antagonist naltrindole (NTI) (Supplementary Fig. 3).

As mentioned previously, cysteine is the rate-limiting precursor for GSH synthesis. Other source of cysteine involves the transsulfuration pathway via homocysteine (HCY). However, in neuronal cells, this pathway is partially blocked, which emphasizes the role of EAAT3-mediated cysteine uptake for GSH homeostasis. HCY can also be converted to methionine via the activity of MS. To investigate whether decreases in cysteine uptake translate into changes in metabolic intermediates involved in transsulfuration and methionine methylation cycle pathways, as illustrated in Supplementary Fig. 4, we measured

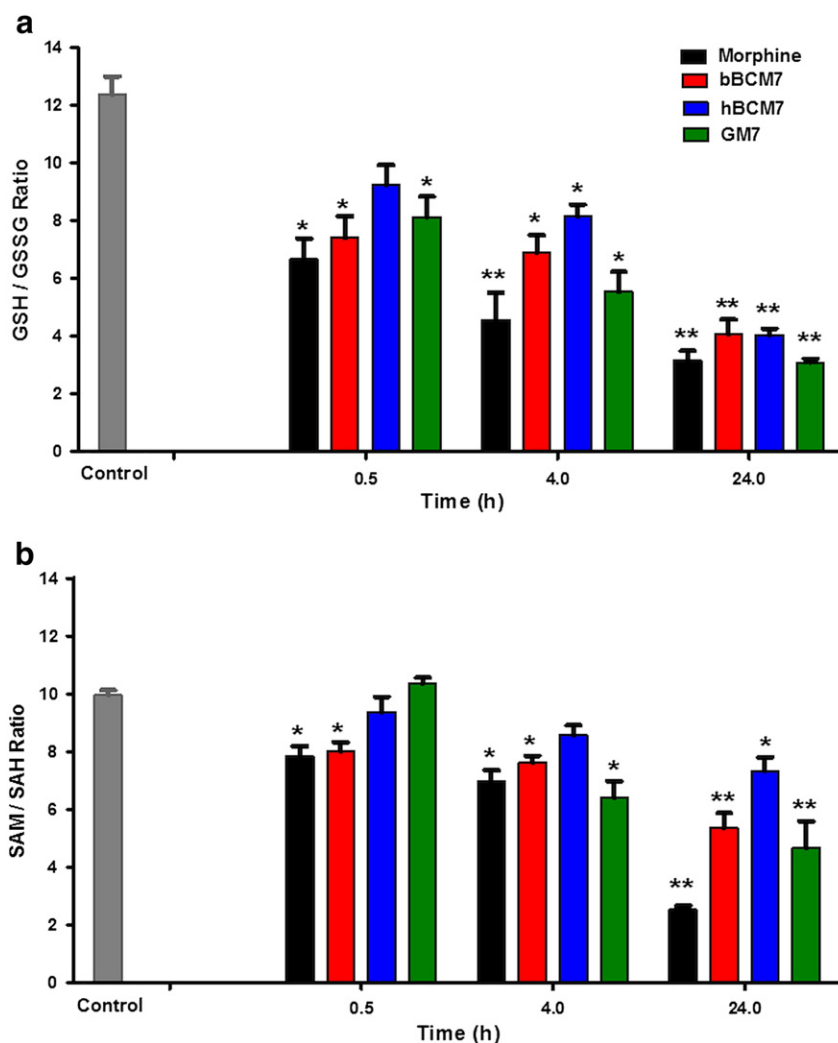


Fig. 2. Effect of opioids on GSH/GSSG and SAM/SAH ratios. Ratios for (a) GSH/GSSG and (b) SAM/SAH were calculated after treatment with opioid peptides and morphine for 0.5, 4 and 24 h, at a concentration of 1  $\mu$ M ( $n=4$ ). \* $P<.05$  and \*\* $P<.01$  vs. control.

their levels in SH-SY5Y cells after treatment with morphine and opioid peptides for different times. Morphine and opioid peptides caused time-dependent decreases in cysteine, GSH and methionine, while HCY and cystathionine levels increased (Supplementary Fig. 5). These changes are consistent with decreased GSH synthesis, decreased activity of MS and increased transsulfuration of HCY to cystathionine.

Redox status is reflected by the ratio of reduced GSH to oxidized GSSG (GSH/GSSG), whereas the ratio of SAM to SAH (SAM/SAH) reflects methylation capacity. Morphine and exorphin peptides caused progressive decreases in GSH/GSSG, reaching more than 3-fold at 24 h ( $P < .01$ , Fig. 2a). Morphine, bBCM7 and GM7, but not hBCM7, transiently decreased SAM/SAH, with a 2–3-fold reduction of SAM/SAH levels observed with morphine, bBCM7 and GM7 treatments at 24 h ( $P < .01$ , Fig. 2b). Thus, the decrease in cysteine uptake caused by morphine and opioid peptides translates into downstream changes affecting redox status and methylation capacity in SH-SY5Y cells.

DNA methylation is an important reaction, representing one of the key mechanisms for epigenetic regulation of gene expression, which has particular significance for postnatal development. We used methyl-CpG binding domain (MBD) protein-enriched genome sequencing (MBD-seq) to evaluate the influence of bBCM7 and hBCM7 on global DNA methylation status in SH-SY5Y cells, compared to the effect of morphine. To evaluate DNA methylation status in the promoter region, 53,561 genes were aligned at their transcription start site (TSS) and the level of methylation was calculated for 3000 bp in either direction, as normalized to the level at the beginning of the region (–3000 bp). As previously described for brain frontal cortex [41], the most prominent feature in the resulting methylation density landscape was an asymmetric peak occurring at about +80 bp, flanked by less methylated valley regions (Fig. 3). A 4-h treatment with morphine or milk-derived opioid peptides (1  $\mu$ M) caused a significant shift toward increased methylation (i.e., promoter hypermethylation) in the immediate TSS region. Morphine was most effective, increasing genome-wide methylation at the TSS peak to 22% above control levels, followed by bBCM7 and hBCM7, representing increases of 17% and 10%, respectively, a rank order that mirrors their inhibition of cysteine uptake. Morphine treatment significantly altered methylation status of a total of 5366 genes in this TSS region, bBCM7 3891 and hBCM7 1627 ( $P < .05$ ). Thus, opioid-induced changes

in cysteine uptake, redox status as well as the SAM/SAH ratios, are associated with significant genome-wide changes in DNA methylation levels at the TSS.

We have previously showed changes in transcription of a panel of 11 redox and methylation pathway genes in association with decreased EAAT3-mediated cysteine uptake in response to the oligomeric A $\beta$  amyloid peptide, implicated in causation of Alzheimer's disease [39]. Hence, we wanted to investigate whether morphine and opioid peptides could induce similar changes in the transcriptional levels of these genes. For this purpose, we used qRT-PCR to evaluate changes in mRNA expression levels after treatment of SH-SY5Y cells with morphine or opioid peptides (4 h, 1  $\mu$ M). hBCM7 significantly increased expression of nine genes in the panel, including EAAT3, GCLC, GCLM, CGL, CBS, MTR, MAT2A, DRD4 and LINE-1 (Fig. 4). In contrast, morphine and bBCM7 decreased the expression of these genes (with the exception of GCLM), while GM7 produced a mixed pattern of increased and decreased gene expression. Thus, food-derived opioid peptides affect redox/methylation gene expression, with hBCM7 and bBCM7 showing opposite effects. These results indicate that morphine and food-derived opioid peptides induce changes in the redox and methylation capacity of the cells by modulating cysteine uptake via EAAT3 and subsequent changes in DNA methylation and gene expression levels were observed. However, the changes in the DNA methylation status might not be directly responsible for changes in the transcriptional status of genes involved in the regulation of redox and methylation pathways, and other mechanisms might be involved in regulating mRNA levels (e.g., transcription factors or mRNA stability).

#### 4. Discussion

Food-derived opioid peptides are produced in the gut, where they can exert effects on the gut intestinal epithelium, but they can also enter the systemic circulation [42] and may be able to cross the blood–brain barrier [43]. Recently, it has also been revealed that casein-derived opioid peptides are present in the urine of autistic and schizophrenic subjects, but not of controls [44,45]. It has been suggested that increased blood levels of bBCM7, in conjunction with lower activity of dipeptidylpeptidase IV, the enzyme responsible for

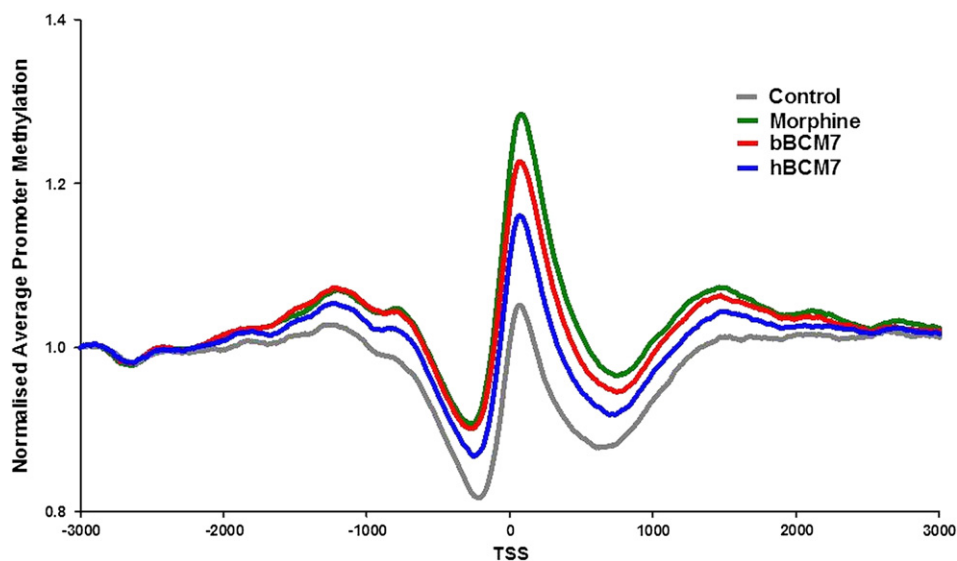


Fig. 3. Morphine and milk-derived opioid peptides increase genome-wide promoter methylation. SH-SY5Y cells were treated with 1  $\mu$ M morphine, bBCM7 or hBCM7 for 4 h ( $n=5$ ), and genome-wide DNA methylation was analyzed by MBD-seq. We aligned 53,561 genes at their TSS, and average methylation between –3000 bp and +3000 bp was computed and normalized to values at –3000 bp. Inset: Methylation of all genes was evaluated at the +80 bp peak for each group and expressed as a percentage of the control group level, yielding  $P$ -values for morphine, bBCM7 and hBCM7 of  $1.8e-25$ ,  $0.016$  and  $3.2e-40$ , respectively.

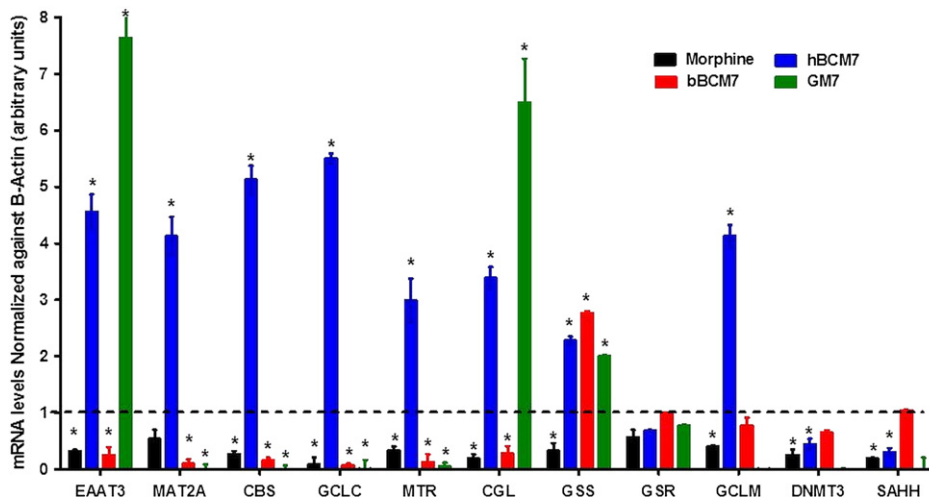


Fig. 4. Morphine and opioid peptides alter redox/methylation gene expression. SH-SY5Y cells were treated with opioid peptides or morphine (1 μM) for 4 h, RNA was isolated and mRNA levels were probed by qRT-PCR with primers designed for a panel of redox and methylation-linked genes. Values were normalized to β-actin levels and to control gene expression (n=4). The y-axis indicates fold changes in mRNA levels. Asterisk (\*) indicates significant difference (P<.05) from untreated control cells.

its hydrolysis, may be associated with apnea in sudden infant death syndrome [45], reflecting μ-receptor-mediated respiratory depression. The current study supports previous results indicating the biochemical effects of the food-derived peptides specifically on the μ-opioid receptor.

Intolerance to casein or gliadin can contribute to inflammation of the gastrointestinal (GI) tract as exemplified by celiac disease, as well as systemic effects including neurological manifestations. While true IgE-based allergy can be triggered by gluten in some individuals, intolerance associated with actions of gluten-derived peptides such as GM7 may affect a larger population, and there is evidence that the number of people suffering from gluten intolerance is increasing [46]. A gluten-free diet usually relieves the symptoms of celiac disease. As mentioned previously, plasma and brain GSH is decreased and other thiol metabolites are abnormal in subjects suffering from autism [8], schizophrenia [7] and celiac disease. In our study, we observed similar pattern of decreased GSH as well as altered thiol metabolites after exposure to morphine and opioid peptides. In light of the above-mentioned clinical evidence and metabolic relationships, data from the current study suggest that the salutary effects of a gluten-free/casein-free diet might reflect actions of the opioid peptides released from milk and wheat on biochemical pathways via modulating the cellular redox status.

Only anecdotal evidence is available for the nutritional and dietary interventions recommended for treatment of ASD, schizophrenia and celiac disease patients, and supporting mechanistic or biochemical investigation is rarely performed. The current study is the first to describe the ability of food-derived opioid peptides and morphine to modulate cysteine uptake, with subsequent effects on cellular redox and methylation status leading to global changes in DNA methylation and gene transcription. As summarized in Fig. 5, these actions constitute a biochemical pathway, namely a redox/methylation signaling pathway, which extends our current understanding of the physiological and pharmacological effects of food-derived opioid peptides. In individuals who are sensitive to milk or wheat, these actions of food-derived opioid peptides may contribute to inflammation and, in the case of milk-derived hBCM7 and bBCM7, they may influence epigenetic programming during early postnatal development period. Systemic effects on redox, methylation and epigenetic status could potentially result either from an inhibitory influence of opioid peptides on cysteine absorption from the GI tract. Additionally, distinctive epigenetic effects of hBCM7 vs. bBCM7 might also explain

the well-recognized health benefits of breast feeding vs. formula feeding.

Changes in epigenetic marks such as DNA methylation are crucial for adaptive changes in gene transcription and early-life experience; for example, nutrition can influence the lifelong risk of diseases such as cancer, type 2 diabetes mellitus, obesity, inflammation and neurocognitive disorders [21–23,47]. Our findings provide a candidate mechanism by which nutritional and dietary components can affect biochemical pathways and induce changes in epigenetic status and subsequently modulate disease risk. Since EAAT3 is expressed in T-cells, including T-regulatory cells [48], this novel μ-receptor-mediated redox-based signaling pathway may also be important for

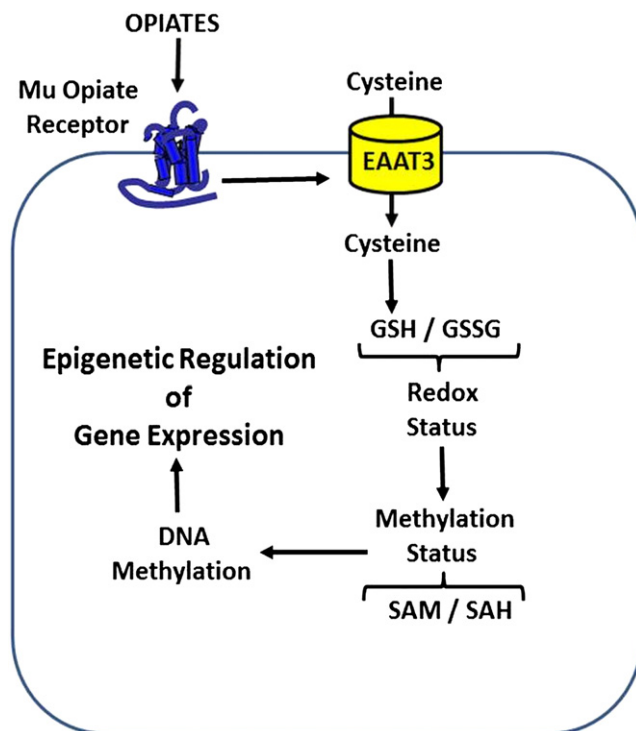


Fig. 5. Summary diagram of redox-dependent epigenetic regulation by μ-opioid receptors.

the immunomodulatory effects of opiate antagonists, such as naltrexone [49]. While the GI tract may be the primary locus for opiate peptide effects, their influence can extend throughout the body, by virtue of their influence over systemic antioxidant resources. It should be noted that bovine form of BCM7 is only released from cows with the A1 genotype and not A2 genotype cows.

While the mechanism underlying inhibition of cysteine uptake by food-derived opiate peptides is not fully established, we previously found that protein kinase A and/or mitogen-activated protein kinase are involved in mediating the effects of morphine on EAAT3 [50]. Their inhibitory effects on cysteine uptake are also observed at the levels of redox status and methylation capacity as well as on DNA methylation status and the transcription of genes involved in maintaining the redox methylation homeostasis. However, the current results do not propose a direct causal relationship between the changes in DNA methylation and the resultant transcriptional state, and several contributing factors, other than the global DNA methylation status, might be affected by opioid peptides. Thus changes in redox and methylation could potentially influence a wide array of factors affecting mRNA levels, including the availability and binding of transcriptional factors or polycomb group proteins, histone modifications or changes in mRNA stability.

It should be noted that the current study was carried out using SH-SY5Y and Caco2 cancer cell lines, which might not reflect responses in non-transformed cells. However, these cell lines have been used previously for epigenetic studies [50,51] and they provide a useful preliminary indication of opiate peptide effects, which needs to be replicated in other systems before being confidently extrapolated to clinical manifestations. However, the current study does provide the first mechanistic explanation through which nutritional factors might impinge on the epigenetic and transcriptional status of genes. The relationship between gluten- and casein-derived opiate peptide-induced redox and methylation changes and downstream effects of these changes still remain to be elucidated. Taken together, our findings provide a novel antioxidant-based biochemical pathway linking gut and brain function via the diet.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2014.05.004>.

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